IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Dkt. No: 5593-00300

Inventor(s):
Bruno Paquin

Title: PROCESS FOR THE GENERATION OF OLIGONUCLEOTIDE LIBRARIES (OLS) REPRESENTATIVE OF GENOMES OR EXPRESSED mRNAs (cDNAs) AND USES THEREOF

CERTIFICATE OF EXPRESS MAIL
UNDER 37 C.F.R. §1.10

"Express Mail" mailing label number: EL914623138US
DATE OF DEPOSIT: July 6, 2001

I hereby certify that this paper (along with any paper and/or fee referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to:
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Derrick Brown

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371

INTERNATIONAL APPLICATION NO.: PCT/CA00/00047
INTERNATIONAL FILING DATE: January 19, 2000
PRIORITY DATE CLAIMED: January 19, 1999
U.S. APPLICATION NO. (If known):

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.

2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.

3. ☐ This is an express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(l).

4. ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)), including: a title page; _____ page specification; _____ page(s) of claims (claims -); 1 page abstract.

5. ☐ Drawings
☐ Formal Figure(s) _____ - _____ on 1 sheet(s).

6. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
☒ is transmitted herewith (39 pages).
☐ has been transmitted by the International Bureau.
☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3))
   - ☒ are transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ have been transmitted by the International Bureau.
   - ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).

9. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
   - ☒ A copy of the Demand for International Preliminary Examination is enclosed.

10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. § 371(c)(4)):
   - ☐ is enclosed (_____ pages).
   - ☐ a combined Declaration and Power of Attorney is enclosed (_____ pages).
   - ☒ is not enclosed. Applicant requests the Patent and Trademark Office to accept this application and accord a serial number and filing date as of the date this application is deposited with the U.S. Postal Service for Express Mail. Further, Applicant requests that the NOTICE OF MISSING PARTS-FILING DATE GRANTED be sent to the undersigned representative of Applicant.

11. ☒ Applicant hereby claims priority to:
   - ☒ International Application No.: PCT/CA00/00047 filed January 19, 2000.
   - ☒ Canadian application No.: 2,259,745 filed January 19, 1999.

12. ☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

13. ☒ The entire disclosure of the International Application referred to above is considered to be part of the accompanying application and is hereby incorporated by reference herein.

14. ☐ Assignment Papers.
   - ☐ An assignment document is enclosed for recording (_____ pages).
   - ☐ Form PTO-1595 Assignment Recordation Cover Sheet (page).

15. ☒ A Preliminary Amendment (8 pages).

16. ☐ A substitute specification for pages _____ (_____ pages).

17. ☐ Power of Attorney
   - ☐ Is enclosed.
   - ☐ a combined Declaration and Power of Attorney is enclosed.

18. ☐ Information Disclosure Statement (IDS), including:
   - ☐ Form PTO-1449
   - ☐ Reference(s) marked according to Form PTO-1449.

19. ☒ Return Receipt Postcard

20. ☒ Small Entity Status
   - ☐ A small entity statement is enclosed.

21 ☒ Copy of International Request.

22. ☒ Copy of International Preliminary Examination Report.
   - ☒ A copy of the International Preliminary Examination Report in English.
   - ☐ English Translation of the International Preliminary Examination Report.

23. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR § 1.492 (a) (1)-(5): ☐ Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO.....$970.00 ☒ International preliminary examination fee not paid to USPTO but International Search Report prepared by the EPO or JPO.....$840.00 ☐ International preliminary examination fee not paid to USPTO but international search fee paid to USPTO.....$760.00 ☐ International preliminary examination fee paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....$670.00 ☐ International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....$96.00	
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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	4 - 20 =	0	x $18.00 =	
Independent claims	1 - 3 =	0	x $78.00 =	
MULTIPLE DEPENDENT CLAIM(S)			+ $260.00 =	

TOTAL OF ABOVE CALCULATIONS:	$840.00
Reduction by 50% for Small Entity. A Small Entity Statement must be filed:	$420.00
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☐ A check in the amount of $_____.00 is enclosed.

☒ Please charge my Deposit Account No. 50-1505/5593-00300 in the amount of $420.00 to cover the above fees.

☐ The Commissioner is hereby authorized to charge any other fees which may be required or credit any overpayment to Conley, Rose, & Tayon, P.C., Deposit Account No. 50-1505/ /EBM.

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Name Mark R. DeLuca
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Date July 6, 2001

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.: Unknown §
Filed: Herewith §
Inventor(s): §
Bruno Paquin §
§
§
§
§
Title: PROCESS FOR THE §
GENERATION OF §
OLIGONUCLEOTIDE §
LIBRARIES (OLs) §
REPRESENTATIVE OF §
GENOMES OR §
EXPRESSED mRNAs §
(cDNAs) AND USES §
THEREOF

Examiner: Unknown
Group/Art Unit: Unknown
Atty. Dkt. No: 5593-00300

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Commissioner for Patents
Box Patent Application
Washington, DC 20231

Derrick Brown

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Amendment

Sir:

Please amend the above-captioned application as follows:

In the Claims:

Please amend the claims as follows. Applicant has appended strikethrough versions of the amended claims to the end of this response.

1. (Amended) A process for generating a library of oligonucleotides that are specific for a given

set of nucleic acids, comprising:

a) generating random oligonucleotides, wherein said oligonucleotides are of a uniform length comprising a single stranded, central segment of randomly varied bases and flanking segments of defined sequences on each side of said central segment;

b) hybridizing the random oligonucleotides with a nucleic acid-containing template of biological or synthetic origin under hybridization conditions that enable the formation of duplexes and using blockers to avoid hybridization of said flanking segments;

c) eliminating non-specific duplexes using conditions that minimize or abrogate mismatches;

d) separating the hybridized oligonucleotides from the duplexes obtained in step c); and

e) amplifying the hybridized oligonucleotides.

2. (Amended) A process as defined in claim 1, further comprising subtracting between two different oligonucleotide libraries (OL1 and OL2) which contain similar sequence motifs.

3. (Amended) A process as defined in claim 2, wherein said subtracting comprises:

a) generating single stranded versions of OL1 and OL2;

b) annealing the OL1 strands with an excess of OL2 strands, under hybridization conditions;

c) partitioning double stranded hybrids (OL1:OL2) and single stranded OL2 from single stranded OL1;

d) amplifying the single stranded OL1; and

e) repeating steps a) to d) to obtain OL1 oligonucleotides with reduced affinity for OL2.

4. (Amended) A process as defined in claim 1, wherein said central segment comprises 10-40 bases and each one of said flanking segments comprises 10-40 bases.

6. (Amended) A process as defined in claim 1, wherein the nucleic acid-containing template comprises at least one of genomic or synthetic DNA or RNA, or cDNA.

8. (Amended) A library of oligonucleotides produced by the process comprising:

a) generating random oligonucleotides, wherein said oligonucleotides are of a uniform length comprising a single stranded, central segment of randomly varied bases and flanking segments of defined sequences on each side of said central segment;

b) hybridizing the random oligonucleotides with a nucleic acid-containing template of biological or synthetic origin under hybridization conditions that enable the formation of duplexes and using blockers to avoid hybridization of said flanking segments;

c) eliminating non-specific duplexes using conditions that minimize or abrogate mismatches;

d) separating the hybridized oligonucleotides from the duplexes obtained in step c); and

e) amplifying the hybridized oligonucleotides.

9. (Amended) Use of a library of oligonucleotides produced by the process of claim 1 in a diagnostic kit.

10. (Amended) Use of a library of oligonucleotides produced by the process of claim 1 to inhibit gene function.

11. (Amended) A method of diagnosis comprising use of a library of oligonucleotides produced by the process of claim 1.

12. (Amended) Use of a library of oligonucleotides produced by the process of claim 1 wherein said oligonucleotides are bound to a solid support.

13. (Amended) A use as defined in claim 12, wherein the solid support is at least one of a membrane, glass slide, coated glass slide, printed arrays, microspheres or chromatographic media.

14. (Amended) Use of a library of oligonucleotides produced by the process of claim 1, wherein said oligonucleotides are hybridized to nucleic acid arrays.

Bruno Paquin

It is believed that no fees are due in connection with the filing of this Preliminary Amendment. However, if any fees are due, the Assistant Commissioner is hereby authorized to deduct said fees from Conley, Rose & Tayon Deposit Account No. 50-1505/5679-00101/EBM.

Respectfully submitted,

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Date: July 6, 2001

Bruno Paquin

STRIKETHROUGH VERSION OF AMENDED CLAIMS

1. A process for generating a library of oligonucleotides that are specific for a given set of nucleic acids, comprising:

a) generating random oligonucleotides, wherein said oligonucleotides are of a uniform length comprising a single stranded, central segment of randomly varied bases and flanking segments of defined sequences on each side of said central segment;

b) hybridizing the random oligonucleotides [of step a)] with a nucleic acid-containing template of biological or synthetic origin under hybridization conditions that enable the formation of duplexes and using blockers to avoid hybridization of said flanking segments;

c) eliminating non-specific duplexes [formed in step b)] using conditions that minimize or abrogate mismatches;

d) separating the hybridized oligonucleotides from the duplexes obtained in [step] c); and

e) amplifying the hybridized oligonucleotides [obtained in step d)].

2. A process as defined in claim 1, further comprising [the step of f)] subtracting between two different oligonucleotide libraries (OL1 and OL2) which contain similar sequence motifs.

3. A process as defined in claim 2, wherein said subtracting [in step f) consists in] comprises:

a) generating single stranded versions of OL1 and OL2;

b) annealing the OL1 strands with an excess of OL2 strands, under hybridization conditions;

c) partitioning double stranded hybrids (OL1:OL2) and single stranded OL2

from single stranded OL1;

d) amplifying the single stranded OL1 [obtained from step c)]; and

e) repeating [steps] a) to d) to obtain OL1 oligonucleotides with reduced affinity for OL2.

4. A process as defined in [any one of claims 1 to 3] claim 1, wherein said central segment comprises 10-40 bases and each one of said flanking segments comprises 10-40 bases.

6. A process as defined in [any one of claims 1 to 3] claim 1, wherein the [template of step b) contains] nucleic acid-containing template comprises at least one of genomic or synthetic DNA or RNA, or cDNA.

8. A library of oligonucleotides produced by the process [of any one of claims 1 to 7] comprising:

a) generating random oligonucleotides, wherein said oligonucleotides are of a uniform length comprising a single stranded, central segment of randomly varied bases and flanking segments of defined sequences on each side of said central segment;

b) hybridizing the random oligonucleotides with a nucleic acid-containing template of biological or synthetic origin under hybridization conditions that enable the formation of duplexes and using blockers to avoid hybridization of said flanking segments;

c) eliminating non-specific duplexes using conditions that minimize or abrogate mismatches;

d) separating the hybridized oligonucleotides from the duplexes obtained in step c); and

e) amplifying the hybridized oligonucleotides.

9. Use of a library of oligonucleotides produced by the process of [any one of claims 1 to 7] claim 1 in a diagnostic kit.

10. Use of a library of oligonucleotides produced by the process of [any one of claims 1 to 7] claim 1 to inhibit gene function.

11. A method of diagnosis comprising use of a library of oligonucleotides produced by the process of [any one of claims 1 to 7] claim 1.

12. Use of a library of oligonucleotides produced by the process of [any one of claims 1 to 7] claim 1 wherein said oligonucleotides are bound to a solid support.

13. A use as defined in claim 12, wherein the solid support is at least one of a membrane, glass slide, coated glass slide, printed arrays, microspheres or chromatographic media.

14. Use of a library of oligonucleotides produced by the process of [any one of claims 1 to 7] claim 1, wherein said oligonucleotides are hybridized to nucleic acid arrays.

TITLE OF THE INVENTION

Process for the Generation of Oligonucleotide Libraries (OLs) Representative of Genomes or Expressed mRNAs (cDNAs) and Uses Thereof

FIELD OF THE INVENTION

The present invention relates to a process for the generation oligonucleotide librairies (OLs) representative of genomes or expressed mRNAs (cDNAs) and to the uses thereof. In particular, the present invention relates to a process for the generation of oligonucleotide librairies comprising oligonucleotides of uniform length. The present invention further relates to the uses of these OLs in numerous biotechnological applications, including the identification and/or characterization of biological materials, clinical diagnosis (DNA/RNA level), preparative extraction of specific mRNA (and genes) and genomic research/mapping.

BACKGROUND OF THE INVENTION

The generation of genomic DNA libraries, or cDNA libraries and the maintenance, and handling of these libraries are critical procedures in the field of genomics and/or biotechnology. In classical libraries the relevant segments of DNA are cloned into vectors, which are maintained and propagated in particular biological systems (*in vivo*). Alternatively, libraries (*in vitro*) can be directly constructed from genomic DNA or cDNA. They contain linkers at the 5' and 3' ends of the DNA which allow PCR amplification of the library. The information stored in these libraries

contains repetitive sequence elements that originated from repetitive DNA, or high copy mRNAs. This results in a significant redundancy, which can complicate the use and the outcome of using classical libraries. Another important feature which reduces the utility of classical libraries is the heterogeneity in size of the members comprising the library. This limits the usefulness of classical libraries in subtractive hybridization procedures (1-2) which are dependent upon the length, complexity and the redundancy of the libraries, and which therefore are particularly sensitive to the choice of method and the number of cycles performed. In fact, one must tailor the hybridization conditions to accommodate the heterogeneous length and redundancy of stored information in order to perform subtraction. Thus, the results are more "laboratory-specific" than library-specific.

A number of diagnostic methods that involve nucleic acid hybridization have arisen in recent years. Most of them are designed to provide qualitative information about the presence of a specific sequence motif in a complex analytical mixture of nucleic acids and use a detection system based on PCR and/or DNA chip hybridization technologies (3-7). For both of these technologies, diagnostic oligonucleotides constitute an essential part of the detection system. These oligonucleotides are primarily chosen based on the sequence data of the nucleic acids to be detected. In spite of the power of hybridization to correctly identify a complementary strand, it does face limitations. In fact, the difference in stability between a perfectly matched complement and a complement mismatched at only one base can be as little as 0.5° C (8). This is the fundamental limitation to the power of DNA hybridization for specific identification of a cognate strand. Therefore, the diagnostic power of any chosen oligonucleotide must be validated using an analytical mixture whose sequence context is

not totally known. The problem of adequate probe selection is time and labour-consuming. On the other hand, the growing complexity of detection systems based on oligonucleotide technologies requires a fast selection of a large number of short oligonucleotides.

Akopyants *et al* (7) performed subtractive hybridization using bacterial DNAs digested by high-frequency restriction enzymes. The use of such restriction enzymes tends to generate DNA fragments having a broadly similar size, about 500 base pairs. However, the uniformity is not rigorous. Moreover, the library created by these restriction fragments still contains a significant number of redundant sequences; consequently, patches of short polymorphism embedded in homologous sequences are going to be missed when such a library is used.

U.S. Patent No. 5,270,163 (8) teaches a method for the isolation of nucleic acids using high-affinity nucleic acid ligands. This method has been termed the SELEX method (Systematic Evolution of Ligands by Experimental Enrichment) and is based on the use of proteins or small molecules, but not nucleic acids, as targets. The selection of oligonucleotides in the SELEX method relies on the three-dimensional (3D) shape of the oligonucleotides and their fit into the structures of the target molecules. In contrast to this, the selection of oligonucleotides in the present invention is based on hybridization with target nucleic acid.

Armour *et al* (11) describes the quantitative recovery of amplifiable probes hybridised to an immobilised target. The amplifiable probes consist of PCR or restriction fragments and their technique is meant to assess the copy number of *loci*.

There thus remains a need for oligonucleotide libraries which allow for the use of uniform hybridization conditions to perform selection and/or subtraction while minimizing or eliminating redundant sequences. Advantageously, these libraries can be used in the selection of highly informative and target-specific probe libraries. The present invention seeks to meet these and other needs.

SUMMARY OF THE INVENTION

The procedure described herein results in the generation and selection of oligonucleotide probes with a high specificity for a given system. These oligonucleotides cover the entire length of the target DNA, thus increasing detectability which might be lost in classical oligo-detection systems due to secondary DNA structure or DNA deletions present in an analyte mixture. At the same time, they present inexpensive variants of a multiplex oligonucleotide-detection approach, since they are not required to be individually synthesized.

More specifically, in accordance with the present invention, there is provided a process for the generation of oligonucleotide libraries, or OLs. The present invention teaches a process for generating OLs from genomic DNAs and cDNAs, and for performing the subtraction of these libraries.

The present invention further teaches OLs which allow the use of hybridization conditions which are controllable and reproducible. In addition, the invention teaches a process for the selection of uniform length OLs which minimizes or eliminates redundant sequences and reduces complexity. The result is the production of highly-informative and target-specific probe libraries.

An object of the present invention is therefore to provide a process for the generation of oligonucleotide libraries comprising OLs of uniform length which are self-amplifiable and easily subjected to subtraction.

Another object of the invention is to provide OLs which are compatible with DNA array technology. Indeed, an array of diverse mixtures of oligonucleotides which show differential hybridization patterns could be the best choice for the next generation of DNA diagnostics.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic representation of the experimental procedure for the preparation of OL. Denatured DNA is bound to the membrane and hybridized to the random oligonucleotides library in the presence of blockers. Theses blocking primers disallow the unspecific hybridization of left and right oligonucleotide arms used for PCR amplification of the OL. (ss - single-stranded DNA, ds - double-stranded DNA). Hybridization and PCR amplification of OL are described more particular below, in the Experimental Methods.

Figure 2: Dot blot hybridization of OL targeted against different genomes. The first row represents the dot blot hybridization of random probes with the specified genomic DNA (adenovirus, pBluescript and lambda). The

last row shows dot blot hybridization of mixed adenovirus and lambda-selected OL. The other rows are analytical dot blot hybridizations of selected OLs with each of the genomes indicated. The procedures of preparative and analytical hybridization are described in the Experimental Methods, below.

Figure 3: Specificity and probe distribution of OL generated from adenoviral genome. (**A**) The corresponding genome and adenoviral DNA were run on a 1% agarose gel stained with ethidium bromide. The type of restriction enzyme and DNA are indicated on the top of each gel lane. (**B**) Southern hybridization of the same gel using adenovirus OL as an hybridization probe (see Figure 2, row 2). It should be noted that under the experimental conditions, there was no cross-hybridization with either lambda or human DNA. (**C**) The same membrane was stripped and rehybridized with a OL directed against a 3648 bp-long restriction fragment. This subset of adenovirus OL was prepared by cutting the membrane corresponding to the 3648 bp band from a similar southern blot and reamplified by PCR as described in the Experimental Methods, below. Thus, it is shown that OL specificity may be enhanced by controlling the choice of targeted DNA fragments in the next round of selection.

Figure 4: The distribution of OL along genomic DNA. The densitrometric scan of radioactive signal from OL was integrated over total adenoviral genome (Figure 3, lanes 3 and 5) using Scion Image software (Scion corporation, Frederick, Maryland). The signal intensity of OL probes hybridizing to restriction fragments is linearly proportional to the length of DNA.

Figure 5: Subtractive enrichment of OL. (**A**) The tester OL is presented by the mixture of two genomes (Adenovirus type 2 and Lambda phage). The driver OL was produced from the Lambda genome only. The single stranded (ss) OL from driver DNA was used to pool out the complementary single stranded mixed tester OL. After removing the subtracted fraction, the remainder of the mixed OL was used as a probe in the analytical hybridization step. The mixed OL probes were analyzed by dot blotting as described (**B**) before subtractive enrichment and (**C**) after subtractive enrichment by hybridization to genomic Adenovirus and Lambda DNA.

Figure 6: Relative distribution of 20-mers with the different number of mismatches which do hybridize to targeted DNA. The abscissa shows the number of mismatches present in the 20-mer, while the y-axis illustrates the corresponding relative frequencies. The distribution profile was obtained by calculating the number of combinations for each particular number of mismatches which are thermostable at 52° C. The y-axis was normalized to reflect the relative distribution (%) over the total number of captured oligonucleotides (100%). The majority of n-mers captured after the first round of selection will be 20-mers with less then 6 mismatches. This is described further in the Detailed Description, below.

DETAILED DESCRIPTION

The present invention thus provides a process for the generation of oligonucleotide libraries having the following characteristics:

1) A uniform length of about 60 bases, comprising a central segment of about 20 bases randomly varied to represent all possible combinations,

and segments of about 20 bases of a defined sequence flanking the central segment on each side;

2) A uniform number of copies for each sequence motif (consequently, there are no differential hybridization kinetics which could originate from the presence of repetitive DNA); and

3) A melting profile which is characterized by a sharp transition from double stranded to single stranded (or vice versa) oligonucleotides. This is a critical advantage in subtractive hybridization procedures.

The use of these OLs enhances the specificity of hybridization to nucleic acids isolated from various sources, thereby allowing for the preparation of oligonucleotide mixtures useful in the detection and quantification of specific nucleic acids or nucleic acid mixtures.

In one particular embodiment, the starting pool of oligonucleotides is chemically synthesized and consists of a random region of a fixed length (L), flanked by a constant sequence (primer binding sites, PBS). The random oligonucleotide pool covers n copies (n=1,2,3...) of all sequence combinations of length L, i.e. 4^L, which is a total of 10^{12} different sequence motifs for L=20 nucleotides. The basic length of oligonucleotides is long enough to generate uniform sequence motifs for a particular biological system. The complexity of the library (10^{12}) overcomes the complexity of the template (which is usually between 10^4-10^9). The random pool is then hybridized with a nucleic acid template isolated from any selected source and the unbound oligonucleotides are washed away under stringent conditions. The remaining, template-bound oligonucleotides are then subjected to amplification, using PCR or other methods known to those of skill in the art and using primers complementary to the constant

flanking segments, thus producing a library of oligonucleotides capable of selectively hybridizing to nucleic acid templates.

The choice of 20-mer for the length of the oligonucleotide library is not arbitrary but is based on the rationale that the length of the particular sequence motif should be long enough to be unique for even the most complex genomes. The L-mer of length L will be, on average, repeated every 4 power L base pairs. The longer the L, the greater the average distance between 2 identical sequence motifs of length L will be. However, this particular combinatorial approach is at best approximative, and other lengths may be suitable as well. The choice of length will depend on such factors as the length and/or complexity of the genome to be detected and compatibility with current nucleic acid amplification and DNA array technologies.

In another embodiment, the amplification described above is performed with one of the PCR primers being labelled with biotin, providing means for purification of the labelled products with streptavidin-labelled substrates (12) or other similar methods. The amplified mixture of unlabelled oligonucleotides specific to one template is hybridized with labelled mixtures of oligonucleotides selected for specificity to one or several nucleic acid templates, and the unbound material is collected. In this manner, a mixture of nucleotides which is enriched for nucleic acids present in the unlabelled library only can be generated.

The process is based on stringent hybridization. Furthermore, high fidelity hybridization between pools of oligonucleotides and templates (genomic DNA or cDNA) is the basic mode of transfer of genomic

information into OLs. An efficient subtractive hybridization procedure is used to accommodate the features of the aforementioned OLs.

The present invention is illustrated in further detail by the following non-limiting example.

EXAMPLE 1

Generation of OLs, Use Thereof in Subtractive Hybridization to Generate Subtractive Oligonucleotides Libraries (SOLs), and Use of OLs or SOLs in Hybridization Experiments

EXPERIMENTAL METHODS

DNA / oligonucleotides

The starting random DNA pool was synthesised by GIBCO BRL (Burlington, Canada), (RAN), 5'-GCCTGTTGTGAGCCTCCTGTCGAA-N_{20}-TTGAGCGTTTATTCTTGTCTCCC-3'. The corresponding left and right arms were (LEFT) 5'-GCCTGTTGTGAGCCTCCTGTCGAA-3' and (RIGHT) 5'-BioGGGAGACAAGAATAAACGCTCAA-3'. The 5'-end biotinylated oligonucleotides were used to pool out complement strands, using BioMag magnetic particles (PerSeptive Biosystems, Framingham, MA). During preparative hybridization, the left and right arms were blocked by (LEFT) 5'-TTCGACAGGAGGCTCACAACAGGC-3' and (RIGHT) 5'GGGAGACAAGAATAAACGCTCAA-3'. Theses oligonucleotides are termed 'blockers' in the text.

The following genomic DNA was used to produce OL: Adenovirus DNA Type 2, (GIBCO BRL), Lambda DNA cI857 *ind1 Sam 7* (New England Biolabs), pBluescript II SK(+) (Stratagene, San Diego, CA). The Human HeLa DNA used as one control was from Clontech (Palo Alto, CA).

Blotting genomic DNA

The genomic DNA was denatured 2-3 minutes at 95° C and cooled on ice. The nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Piscataway, NJ) was blotted with 100 ng of denatured genomic DNA, dried for 2 minutes on a hot plate and exposed to UV light for 8 minutes. The prehybridization was done for a minimum of 30 minutes in the hybridization buffer (7% SDS, 0.25M Na2HPO4 pH7.4, 1mM EDTA, pH 8.0 and 10g/L of BSA).

Hybridization and washing of the starting random pool

The preparative hybridization between random core (20N) and targeted DNA was done with 10 pmoles of starting random pool (RAN). The random pool was pre-mixed with 100 pmoles (10 times more than RAN) of LEFT and RIGHT blockers in order to exclude cross-hybridization of left and right arms with genomic DNA. The oligonucleotide mixture was heated up to 95°C, cooled at room temperature and added to the hybridization buffer. The hybridization was done overnight at 50° C. The first washing was done with 6X SSC, followed by subsequent 2X SSC washing at the same temperature as hybridization was done.

Generating OL by PCR

The dot containing the genomic DNA and bound probes was cut out of the nylon membrane (radius of 2-4mm), soaked in 100 µl H_20 and heated to 95°C for 1-2 minutes. The solution containing the denatured probe (originally RAN) was then collected and passed threw a Sephadex G-50 column in order to eliminate salts and SDS. The PCR was prepared under standard conditions, typical for SELEX-like amplification of DNA (10, 13). The RIGHT 5'-end biotinylated primer of the sense strand (the

one which did not hybridise with genomic DNA) and LEFT primer of antisense strand were used in the PCR reaction. The temperature cycles were 53°C, 72°C, 95°C, each 30 seconds, repeated 20 times.

Probe labelling and hybridization

Before labelling, the PCR reaction mixtures were passed threw Sephadex G-50 columns. Around 100-200 ng of PCR product was labelled with 50 pmols of γP^{32} ATP (6000 Ci/mmol, I.C.N. pharmaceuticals, Irvine, CA). The total amount of probe radioactivity was 300 000 c.p.m. The probe was added into 0.5 ml of hybridization buffer. The blotting of genomic DNA was done as described above. Hybridizations were done overnight at 50°C. The nylon membrane was washed as previously described, and exposed to Kodak X-OMAT film.

OL labelling and analytical hybridization

The generated OL was tested, using 1) the original genomic DNA from which they were selected (positive control) and 2) using the unrelated genomic DNA (negative control). The OL labelling, hybridization and probe washing was done as described, except that hybridization time was shorter (60 minutes).

Southern blot hybridization

Electrophoresis was performed in a 1% agarose gel with TBE buffer (80 mM Tris borate, pH 8.0, 2mM Na_2EDTA) and stained with ethidium bromide. One ug of BstEII-digested lambda DNA, 300 ng of adenoviral DNA and 1 µg of AluI-HpaI-digested human HeLa DNA were run on the gel according to specifications (all restriction enzymes used in this work were purchased from New England Biolabs). For Southern hybridization, DNA was transferred to Nylon membranes by capillary blot procedure

following manufacturer's recommendations (Amersham Pharmacia Biotech). Hybridization was performed as described above with adenoviral OL. Autoradiographic exposure (using Kodak X-OMAT film) was done at room temperature, for few hours. Stripping of the membrane was done by boiling a 1% SDS solution and pouring it over the Nylon membrane.

Subtractive enrichment of OL

The tester OL (mixed OL) that reflects the two genomes (Adenovirus type 2 and Lambda) was made by preparing OL from equimolar mixtures of 2 genomes. The driver OL was produced from the lambda genome only. The production of sense strand (the one which did not hybridize with genomic DNA) was done using 5'-end biotinylated primer in PCR reaction. After denaturing PCR product, the biotinylated sense strand was bound to streptavidin magnetic particles (200 μg, binding capacity > 200 pmols of biotinylated oligonucleotides, Biomag Magnetic Particles, PerSeptive Biosystems), and pulled-out using a magnet. The complementary antisense strand was discarded with the liquid phase. The mixed antisense tester OL (Lambda + Adenovirus DNA) was produced in the same way. This time, the supernatant with the antisense, non-biotinylated strand was hybridized overnight at 50°C with 10 times molar excess of driver Lambda sense stand attached to magnetic beads. The hybridization buffer was the same as described above but without SDS. After removing the fraction bound to the magnetic beads, the rest of the mixture was used in the analytical hybridization step.

RESULTS

The starting random pool of oligonucleotides contains 4^{20} (i.e. 10^{12}) different 20-mers. The diversity of the sequence motifs is approximately

10^{11} higher than the diversity of the most complex genomes. A schematic representation of the procedure for generating OL is presented in Figure 1 and is described in detail in the Experimental Methods, above.

Blockers were used in order to avoid hybridization of the flanking arms to the targeted genome, and this step was found to be critical to achieve specificity. The stringency of hybridization conditions eliminates unbound 20-mers, leaving the specific oligonucleotides bound to the membrane via hybridization of the random core to the genome (Fig. 1). This ensemble of selected oligonucleotides constitutes the OL.

It should be noted that the starting random pool of oligonucleotides contained about 8 copies of each sequence motif during the first hybridization step (10-20 pmoles) and that the number of copies of each particular 20-mer present in the random mixture was smaller than the number of genome copies.

Figure 2 shows that OLs are able to discriminate genomes with complexities around 10^3 to 10^4. The starting random pool of probes binds to all three genomes equally (Fig. 2, row 1). After one round of selection, the OL can hybridise specifically towards a single targeted genome (Fig. 2, rows 2, 3 and 4). The OL can be selected against a mixture of two genomes and the specificity is conserved for both genomes (Fig. 2, row 5).

A Southern blot was performed in order to document the distribution of adenovirus OL probes along the genome (Fig. 3). There was no apparent cross-hybridization of adenovirus OL to either HeLa or Lambda DNA (Fig. 3b, lanes 1, 4, 5 and 6). The intensity of radioactive signal over adenoviral genome generated by adenovirus-specific OL was linearly increasing with

the DNA fragments' length (Fig. 4). Therefore, one could deduce a uniform distribution of OL throughout the genomic DNA.

In the next step, only the subset of the adenovirus OL bound to the 3648 bp band in Figure 3b (rows 3 and 6) was reamplified and selected. The membrane was washed from the original probe and hybridised with the 3648 bp subset of the original OL. Figure 3c shows that the specificity of the OL subset is obtained against the 3648 bp band. These data illustrate the successful increase in the specificity and the reduction in the complexity of the original Adeno-specific OL to that of the 3648 bp subset, using just one additional round of selection.

One round of subtractive enrichment between two oligonucleotide libraries was performed as schematized (Fig. 5a). The tester OL reflects the two genomes (adenovirus type 2 and lambda phage). The driver OL was produced from the Lambda genome only. The single stranded (ss) OL from the driver DNA was used to pool out the complementary single stranded, mixed, tester OL. After removing the subtracted fraction, the rest of ssDNA was used as a probe in the analytical hybridization step. The intensity of hybridization signals between Lambda and Adeno genomes, before (Fig. 5b) and after (Figure 5c) one round of subtractive enrichment was shown. It should be noted that further subtraction steps could be performed by changing the sequence design of flanking arms between tester and driver OLs, as suggested by recent developments in subtractive procedures (14).

With reference to Figure 6, the relative distribution of 20-mers with different numbers of mismatches that hybridized to the targeted DNA was predicted. The number of 20-mers (N) with (m) number of mismatches

(m=0, 1, ..., 20) capable of hybridizing to the target sequence were then calculated. First, the number of combinations of 20-mers (C) with the same number of mismatches (m) in the initial random pool of oligonucleotides that are capable of hybridizing to a specific 20-mer motif was calculated. Since each full match could be replaced by 3 different mismatches, the number of combinations must be multiplied by 3^m i.e. $C*3^m$. Finally, these numbers were adjusted to reflect the sequence-dependent termostability of 20-mers with (m =0, 1...20) number of mismatches which hybridised at 52°C using the thermostable fraction of the binomial distribution for each n-mer population. Duplexes of more than 7 mismatches are not observed based on this themostability criterion. Therefore, the majority of 20-mers captured after the first round of selection will harbour less than 6 mismatches.

A process that generates amplifiable DNA oligonucleotide libraries which are specific for a given segment of DNA has been described. This process is akin to random priming, because it is possible to generate probes without *a priori* knowledge of the template sequence. One round of preparative hybridization was enough to produce genome-specific oligonucleotide libraries (Figs. 2 and 3). The OLs were inferred from genomes of complexity of 10^3-10^4.

The process described herein generates probes with high detection power. These probes/selected oligonucleotides can contain mismatches. The notion that introduction of artificial mismatches could increase detection power of oligonucleotides during single nucleotide polymorphism (SNP) detection was well documented by Guo *et al* (6). However, the prediction of positions and types of mismatches, which should be introduced to increase detectability of oligonucleotide, remains

undefined. Consequently, to enhance oligonucleotide detectability by introducing (artificial) mismatches, one must search different positions and types of mismatches along the oligonucletide. Once they are empirically determined, i.e. tested on 2 different sequence motifs, the oligonucleotide containing particular mismatches could be used (15). The present process provides an approach based on differential selection of thermostable oligonucleotides (i.e. their differential stability), which are present in one, but not in the second system. The selection of oligonucleotides with the highest detectability is inherently present in this process, i.e. the method suggests a solution to the problem of where and what type of mismatches should be introduced to increase detection power of oligonucleotide, or to find the particular oligonucleotide which best discriminates between 2 sequence motifs which may differ by a single base.

Without wishing to be bound by any hypothesis, the following provides an explanation of what is believed to be occurring during the process of the present invention. Based on calculations, it is expected that the 20-mers selected in an OL can contain up to 6 mismatches. Nevertheless, specificity toward a given template was achieved, suggesting that the presence of these putative mismatches did not interfere with good discrimination. In other words, mismatch-free hybridization is not critical for differential detection approach; rather, the *relative differences* in the thermodynamical stabilities of the hybridized oligonucleotides appear to be determinative. The present process uses selection of oligonucleotides based on this criterion and therefore provides the possibility of overcoming current technological limitations. In the second and further rounds of selection, the number of 20-mers both in the targeted genome and the probe mixture (OL) could be adjusted. Each new round of

preparative hybridization (Fig.1) and/or subtraction (Fig. 6) could reduce the complexity of OL, by using the excess OL rather than genomic DNA. Therefore, the average number of mismatches for each particular 20-mer will continue to decline until it reaches the sequence-dependent limitation, but not the concentration-dependent limitation.

In summary, OLs are generated from the template DNAs. These OLs are used in subtractive hybridization, for example between genomic or cDNA-based libraries (OL1 and OL2) to make a new Subtractive Oligonucleotide Library (SOL1/2 and/or SOL2/1)), that is/are specific for one system/library but not for the other. Oligonucleotides isolated from such subtractive libraries (SOL) are useful for diagnostic purposes. They can a) directly serve as highly specific hybridization probes or b) they can be tested for PCR-specific differential amplification, specific for one, but not the other biological system.

These libraries (OL or SOL) can be hybridized to oligonucleotide chip arrays in order to obtain a specific hybridization pattern that is useful for diagnostic features: each OL produces an image which is specific for the templated DNA (genome or cDNA). A particular advantage in using OL or SOL instead of genomic/cDNA libraries is that the hybridization signal is not dependent on copy number and distribution of particular sequence motifs. By comparing images of different genomes/cDNA, one can deduce which oligonucleotides are highly specific for a single genome/cDNA, and use this or these oligonucleotide(s) as "genome tags". The oligonucleotides obtained can also be used for specific diagnostic PCR.

OLs or SOLs can be inferred from two biologically relevant systems, like mammalian cells, to detect fine differences in cell cycle, tissue status, viral infection, age/development status etc.

Although the present invention has been described hereinabove by way of a preferred embodiment, it can be modified by one of skill in the art without departing from the spirit and nature of the subject invention, as defined more particularly in the appended claims.

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WHAT IS CLAIMED IS:

1. A process for generating a library of oligonucleotides that are specific for a given set of nucleic acids, comprising:

a) generating random oligonucleotides, wherein said oligonucleotides are of a uniform length comprising a single-stranded, central segment of randomly varied bases and flanking segments of defined sequences on each side of said central segment;

b) hybridizing the random oligonucleotides of step a) with a nucleic acid-containing template of biological or synthetic origin under hybridization conditions that enable the formation of duplexes and using blockers to avoid hybridization of said flanking segments;

c) eliminating non-specific duplexes formed in step b) using conditions that minimize or abrogate mismatches;

d) separating the hybridized oligonucleotides from the duplexes obtained in step c); and

e) amplifying the oligonucleotides obtained in step d).

2. A process as defined in claim 1, further comprising the step of

f) subtracting between two different oligonucleotide libraries (OL1 and OL2) which contain similar sequence motifs.

3. A process as defined in claim 2, wherein said subtracting in step f) consists in:

a) Generating single stranded versions of OL1 and OL2;

b) annealing the OL1 strands with an excess of OL2 strands, under hybridization conditions;

c) partitioning double stranded hybrids (OL1:OL2) and single stranded OL2 from single stranded OL1;

d) amplifying the single stranded OL1 obtained from step c); and

e) repeating steps a) to d) to obtain OL1 oligonucleotides with reduced affinity for OL2.

4. A process as defined in any one of claims 1 to 3, wherein said central segment comprises 10-40 bases and each one of said flanking segments comprises 10-40 bases.

5. A process as defined in claim 4, wherein said central segment comprises 20 bases and each one of said flanking segments comprises 20 bases.

6. A process as defined in any one of claims 1 to 3, wherein the template of step b) contains at least one of genomic or synthetic DNA or RNA, or cDNA.

7. A process as defined in claim 3, wherein said partitioning is carried out using streptavidin and biotin.

8. A library of oligonucleotides produced by the process of any one of claims 1 to 7.

9. Use of a library of oligonucleotides produced by the process of any one of claims 1 to 7 in a diagnostic kit.

10. Use of a library of oligonucleotides produced by the process of any one of claims 1 to 7 to inhibit gene function.

11. A method of diagnosis comprising use of a library of oligonucleotides produced by the process of any one of claims 1 to 7.

12. Use of a library of oligonucleotides produced by the process of any one of claims 1 to 7 wherein said oligonucleotides are bound to a solid support.

13. A use as defined in claim 12, wherein said solid support is at least one of a membrane, glass slide, coated glass slide, printed arrays, microspheres or chromatographic media.

14. Use of a library of oligonucleotides produced by the process of any one of claims 1 to 7, wherein said oligonucleotides are hybridized to nucleic acid arrays.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68

A1

(11) International Publication Number: WO 00/43538

(43) International Publication Date: 27 July 2000 (27.07.00)

(21) International Application Number: PCT/CA00/00047

(22) International Filing Date: 19 January 2000 (19.01.00)

(30) Priority Data:
2,259,745 19 January 1999 (19.01.99) CA

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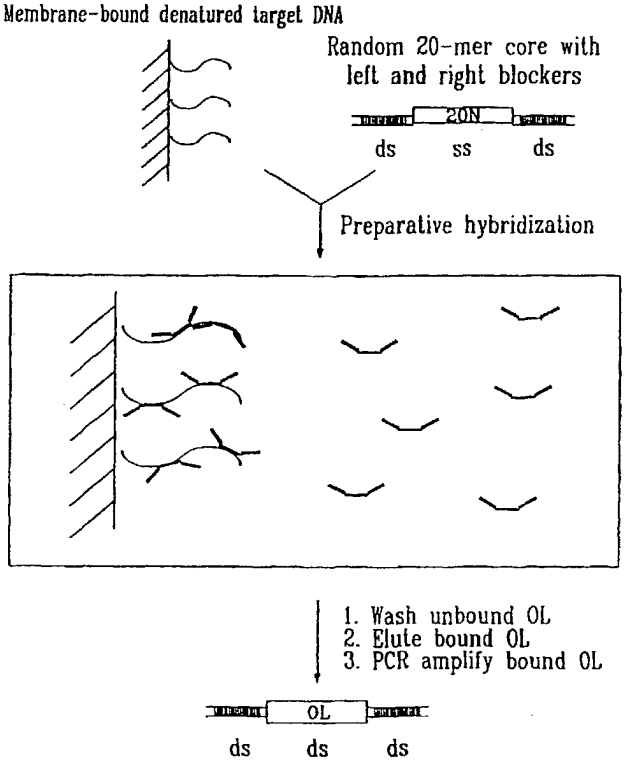
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROCESS FOR THE GENERATION OF OLIGONUCLEOTIDE LIBRARIES (OLs) REPRESENTATIVE OF GENOMES OR EXPRESSED mRNAs (cDNAs) AND USES THEREOF

(57) Abstract

A process for the generation of oligonucleotide libraries representative of a given template is described. Starting from a random pool of oligonucleotides, the process selects only those which hybridize to the template nucleic acid. This selection yields a highly specific library that represents an oligo–image of the chosen template. The novel quality of this approach is the generation of amplifiable oligonucleotide probes that are of uniform length, free of repetitive sequence motifs and easily subjected to differential selection. This technique is used to produce different oligonucleotide libraries (OLs) and shows that these OLs do not cross-hybridize. Differential selection of these OLs produces oligonucleotides that can be used in the identification, characterization and isolation of nucleic acids.



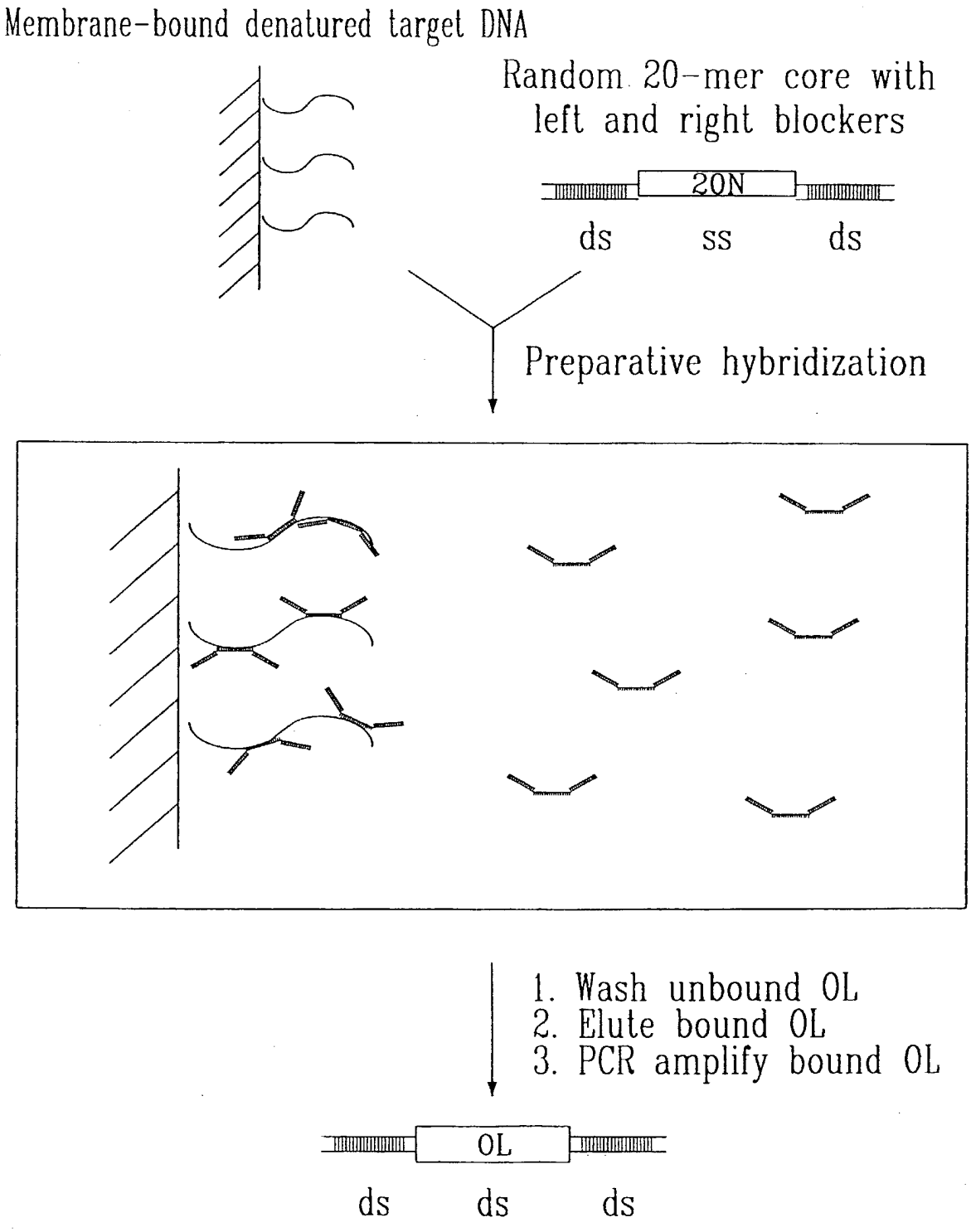

Membrane-bound denatured target DNA
Random 20-mer core with left and right blockers
20N
ds ss ds
Preparative hybridization
1. Wash unbound OL
2. Elute bound OL
3. PCR amplify bound OL
OL
ds ds ds

Fig. 1

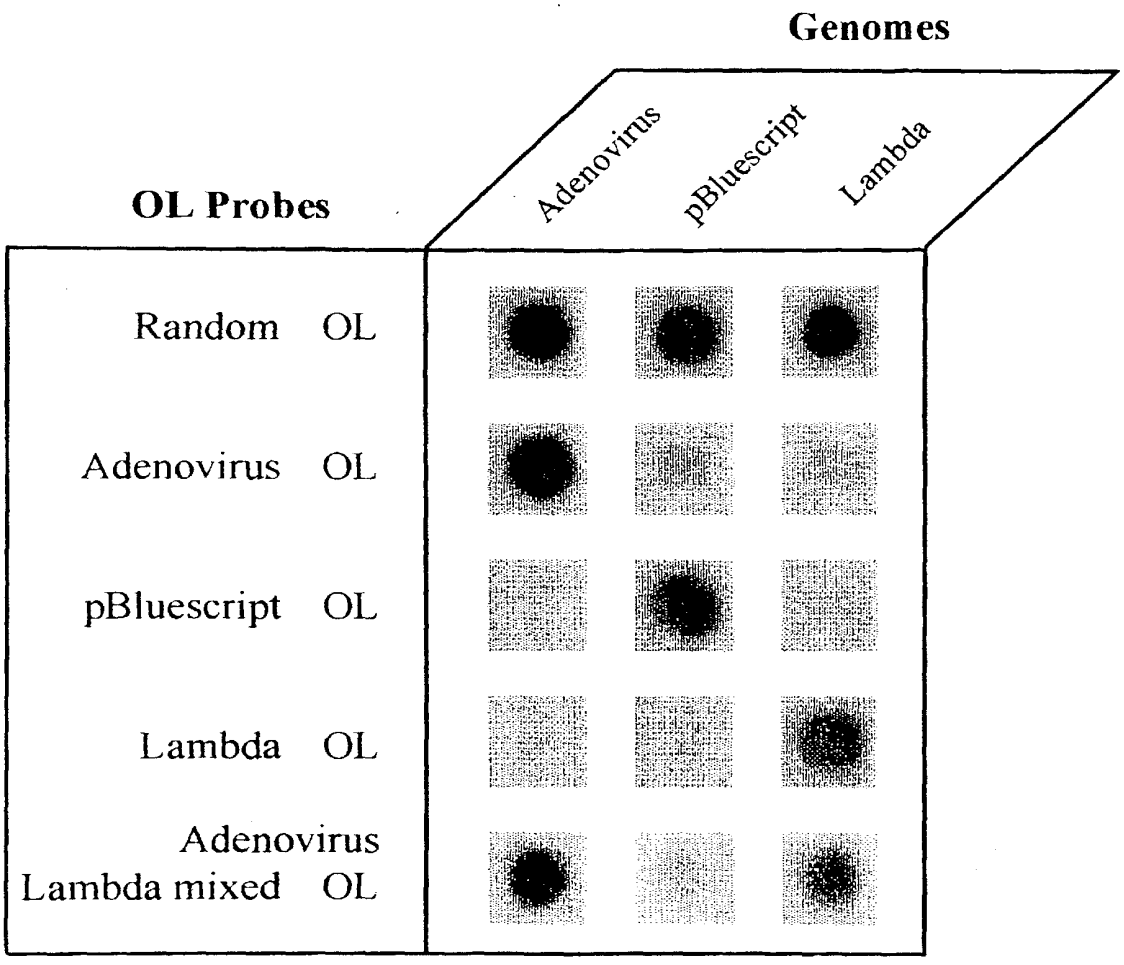

Genomes
Adenovirus
pBluescript
Lambda
OL Probes
Random OL
Adenovirus OL
pBluescript OL
Lambda OL
Adenovirus
Lambda mixed OL

FIG. 2

FIG - 3A

1 2 3 4 5 6
Lambda BstEII
Adenovirus
Adenovirus KpnI
Hela DNA AluI + HpaI
Adenovirus & Hela AluI + HpaI
Adenovirus KpnI & Hela AluI + HpaI

FIG - 3B

1 2 3 4 5 6
Lambda BstEII
Adenovirus
Adenovirus KpnI
Hela DNA AluI + HpaI
Adenovirus & Hela AluI + HpaI
Adenovirus KpnI & Hela AluI + HpaI

FIG - 3C

1 2 3 4 5 6
Lambda BstEII
Adenovirus
Adenovirus KpnI
Hela DNA AluI + HpaI
Adenovirus & Hela AluI + HpaI
Adenovirus KpnI & Hela AluI + HpaI

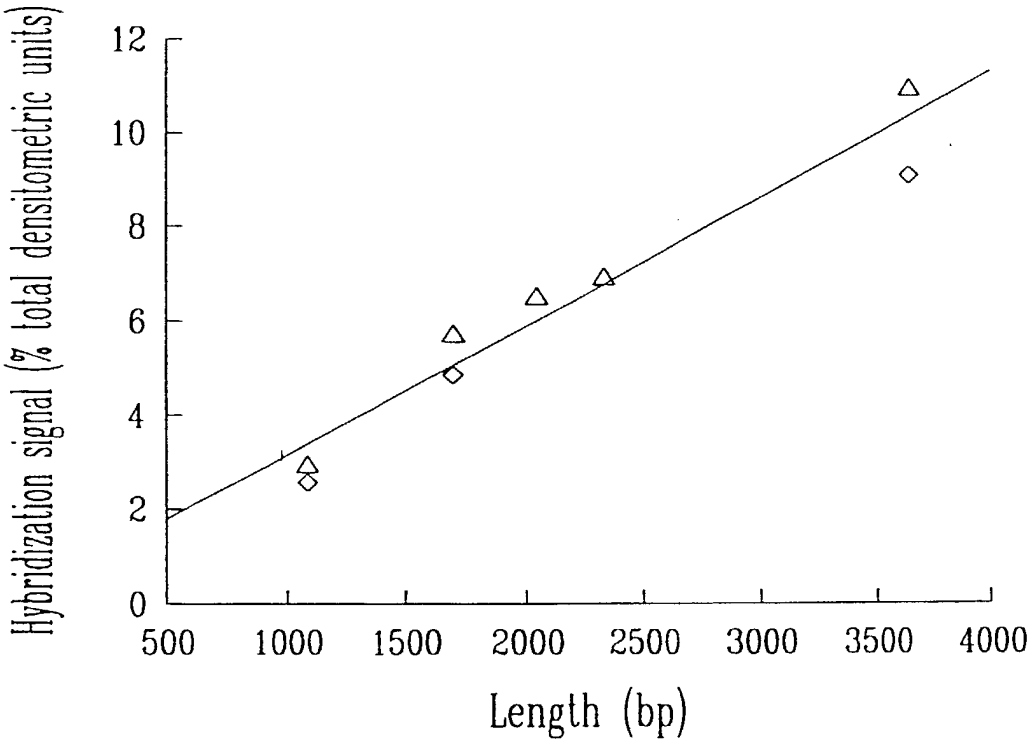

Hybridization signal (% total densitometric units)
0
2
4
6
8
10
12
500
1000
1500
2000
2500
3000
3500
4000
Length (bp)

Fig. 4

5/7

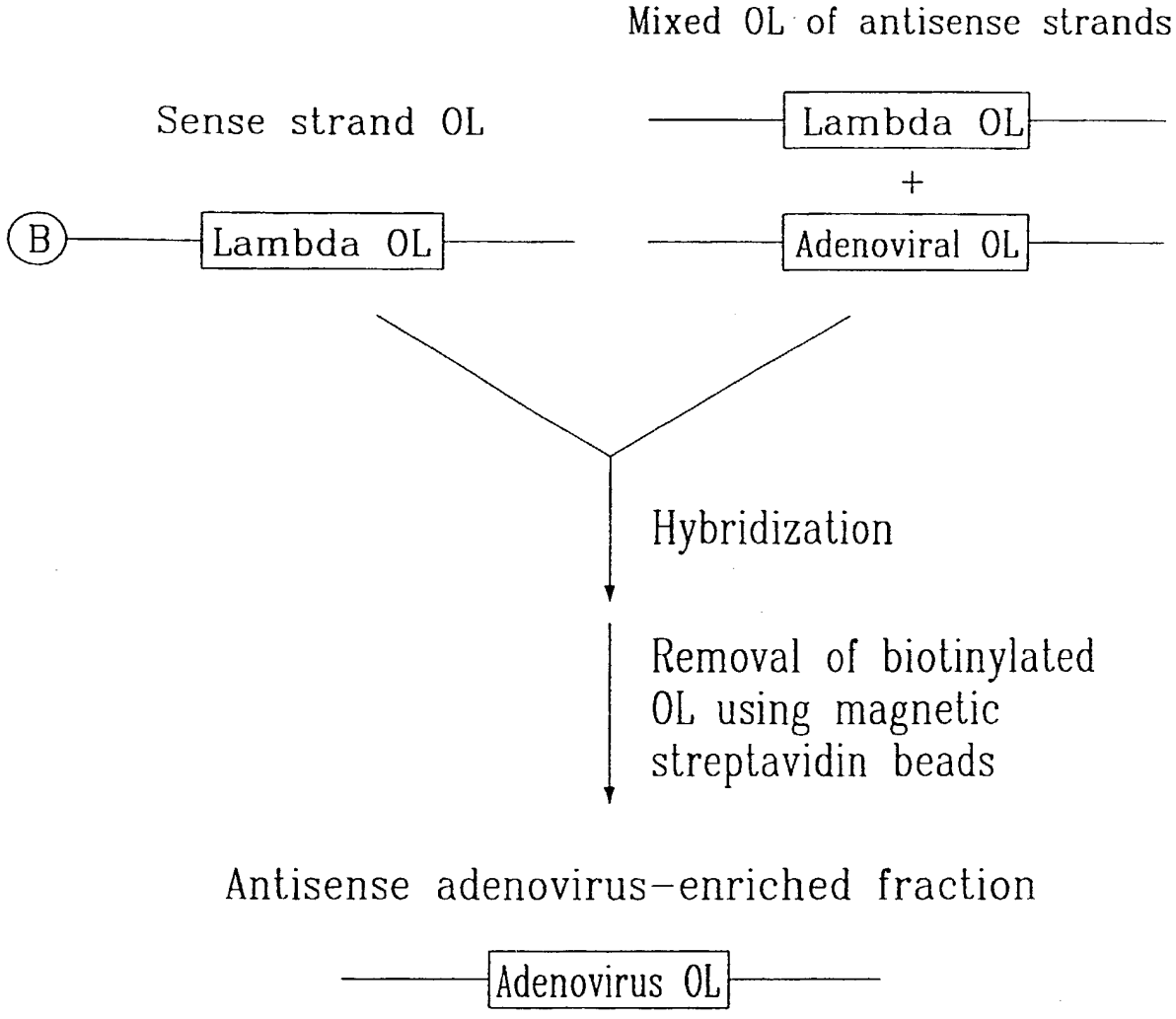

Mixed OL of antisense strands
Sense strand OL
Lambda OL
+
B
Lambda OL
Adenoviral OL
Hybridization
Removal of biotinylated OL using magnetic streptavidin beads
Antisense adenovirus-enriched fraction
Adenovirus OL

Fig 5A

Adenovirus Lambda

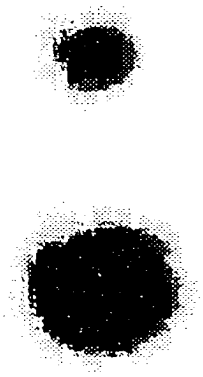


FIG. 5C

Adenovirus Lambda

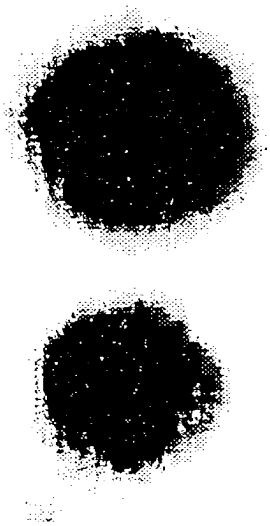


FIG. 5B

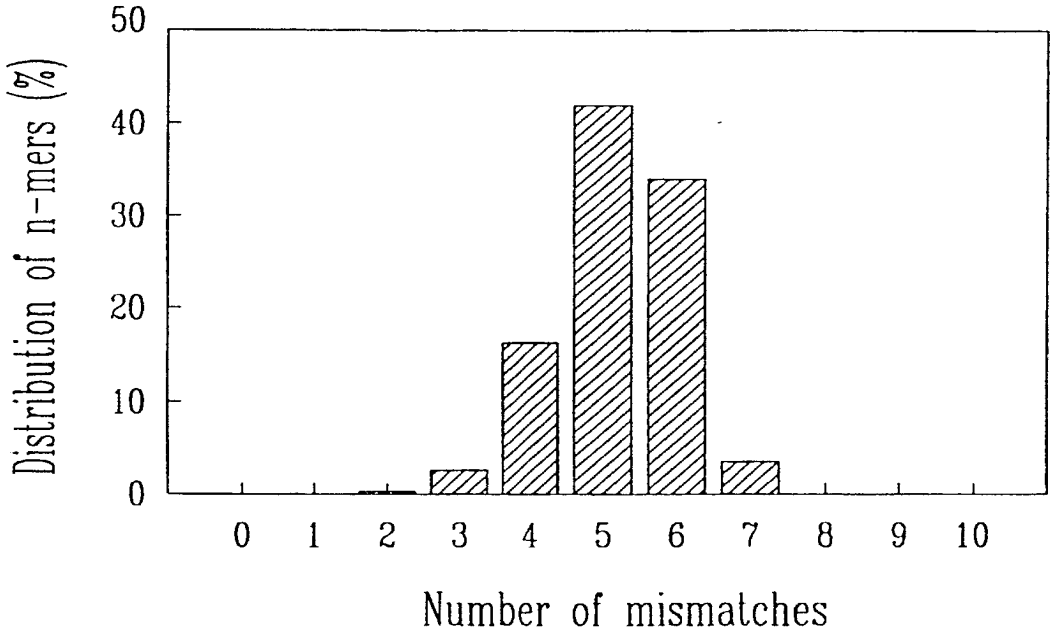

Distribution of n-mers (%)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Number of mismatches

Fig. 6

PATENT

DECLARATION

As a below named inventor, I hereby declare that:

My residence, post office and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or the below named inventors believe they are the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **PROCESS FOR THE GENERATION OF OLIGONUCLEOTIDE LIBRARIES (OLs) REPRESENTATIVE OF GENOMES OR EXPRESSED mRNAs (cDNAs) AND USES THEREOF**, the specification of which:

____ is attached hereto.
X was filed on July 6, 2001 as Application Serial No. 09/869,891 .

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)			Priority Claimed
2,259,745	Canada	January 19, 1999	Yes
(Number)	(Country)	(Date Filed)	
PCT/CA00/00047	PCT	January 19, 2000	Yes
(Number)	(Country)	(Date Filed)	

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose all information known to me to be material to the patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations, § 1.56, which become available between the filing date of the prior application and the national or PCT international filing date of this application:

N/A		
(Application Serial No.)	(Filing Date)	(Status)
N/A		
(Application Serial No.)	(Filing Date)	(Status)

I hereby claim the benefit under title 35, United States code §119(e) of any United States provisional application(s) listed below:

(Application Serial No.) (Filing Date)

(Application Serial No.) (Filing Date)

Please direct all communications as follows:

Eric B. Meyertons
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I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1W

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Country of Residence: Canada Citizenship: Canada

Post Office Address: 3341 Maréchal #4, Montreal, Quebec H3T 1M8 CANADA CA

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PATENT

Applicant or Patentee: Bruno Paquin. Attorney's
Serial or Patent No.: _____ Docket No.: 5593-00300/EBM
Filed or Issued: _____
For: **PROCESS FOR THE GENERATION OF OLIGONUCLEOTIDE LIBRARIES (OLs) REPRESENTATIVE OF GENOMES OR EXPRESSED mRNAs (cDNAs) AND USES THEREOF**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(F) AND 1.27(D)) – NONPROFIT ORGANIZATION

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[X] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: UNIVERSITÉ DE MONTRÉAL
ADDRESS OF CONCERN: Postal Code 6128 Station A
Montreal, Quebec, H3C 3J7 CANADA

I hereby declare that the above identified nonprofit organizati0on qualifies as a nonprofit organization as defined in 37 CFR 1.9(e), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35 United States Code, in that the above identified nonprofit organization is a university or other institution of higher education located in any country.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **PROCESS FOR THE GENERATION OF OLIGONUCLEOTIDE LIBRARIES (OLs) REPRESENTATIVE OF GENOMES OR EXPRESSED mRNAs (cDNAs) AND USES THEREOF** by inventor(s) described in

[ ] the specification filed herewith
[X] application serial no.: 09/869,891, filed July 6, 2001
[ ] patent no.: _____, issued

If the rights held by the above identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME: _____

ADDRESS: _____

[ ] Individual [ ] Small Business Concern [X] Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Jean Yvon Timothy

TITLE OF PERSON OTHER THAN OWNER: Director Industrial Liaison Office and Grants

ADDRESS OF PERSON SIGNING: Same as above

SIGNATURE: _____ DATE: October 22, 2001